IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

EXAMINER:	Gakh, Yelena G.	Docket No.: 4969-A-07
APPLICANT:	Brian L. Craine	Art Unit: 1743
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FILED:	November 26, 2001	
FOR:	METHOD FOR DETERMINING LOCATION OF GASTROINTESTINAL BLEEDING	

DECLARATION OF BRIAN L. CRAINE

UNDER 37 C.F.R. 1.132

The undersigned, Brian L. Craine, makes the following declaration:

1. I am the sole inventor of the above identified application. I also am an owner of Western Research Company, Inc., which is the assignee of the above identified application.

2. My educational background is as follows: I received an M.D. degree in medicine from the University of Miami in 1986, and M.S. and Ph.D. degrees from the University of Texas (Dallas) in 1977, and a B.S. in biology from Wright State University (Dayton, Ohio) in 1973.

3. My professional/employment experience is as follows:

1984 to present, Director of Research, Western Research Company (Tucson, Az);

1987-1990, Resident in Internal Medicine Highland General Hospital (Oakland, CA);

1986-1987, Intern in Internal Medicine (Highland General Hospital(Oakland, CA);

1981-1984, Research Scientist, Chiron Corporation (Emeryville, CA);
1977-1981, Postdoctoral fellow, University of California, San Francisco (San Francisco Ca).

4. I have reviewed the Office Action of 12-15-2003 and cited references. I also have reviewed the accompanying amendment, and I agree with the statements and arguments made therein.

5. A person skilled in the art of medicine and medical research regarding occult bleeding wanting to measure a test sample using spectroscopy almost always first obtains a standardized spectrophotometric reading by using reference samples which are known forms of the unknown test sample.

6. A person skilled in this art wanting to perform the method of the claimed invention in accordance with the description provided in the specification would ordinarily prepare reference samples having the same pH as the stool test sample and would use the spectrophotometer to determine the peak values of the main Soret band and also to determine any visible additional absorption peaks of both pure hemoglobin (ferrous heme) and acid-treated hemoglobin (ferric heme) before using the same spectrophotometer to determine the values of

corresponding peaks in the spectrum of the stool test sample.

7. A person skilled in this art wanting to perform the method of the claimed invention in accordance with the specification could and ordinarily would easily prepare the reference samples in essentially the same manner described on page 15, lines 9-18 of the specification for preparing the stool test sample, except that the first reference sample would be prepared by using pure hemoglobin instead of fecal matter and adjusting the pH to the same value as that of the stool test sample in order to determine the absorption peak of the main Soret band for pure hemoglobin (ferrous heme) and any associated additional visible peaks. The person skilled in the art could also easily prepare a second reference sample using hemoglobin treated with acid similar to that present in the human stomach and adjusting the pH of the second sample to the same value as the pH of the stool test sample in order to determine the absorption peak of the main Soret band and any additional visible peaks for the acid-treated hemoglobin (ferric heme).

8. I obtained the reference spectra shown in Fig. 2 of the above identified patent application and described in the specification on page 14, lines 10-18. That is how anyone skilled in this art wanting to perform the method of invention could have done it. In my opinion, it would have been pointless to run numerous experiments at different pH levels to provide data absorption peaks as a function of pH, because anyone wanting to practice the invention would ordinarily have prepared and then spectroscopically analyzed corresponding reference samples as a matter of ordinary good spectroscopic analysis practice.

9. The changes in the locations of the absorption peaks of ferrous heme and ferric heme are small over the meaningful pH range, which are indicated below in Table 1. However, the small changes in the locations of the absorption peaks of ferrous heme and ferric heme ordinarily would be irrelevant because the usual procedure would be to measure "standard" ferrous heme preparations and ferric heme preparations under the exact same conditions as the stool sample to be tested. In a commercial version of the invention, a small tube of a "standard" ferrous heme preparation and a small tube of a standard ferric heme preparation could be provided for the user's convenience in preparing "standard" ferrous heme and ferric heme preparations having a pH corresponding to that of a particular buffer desired by the user for preparing a test stool sample, and then measuring, i.e., "standardizing", the ferric heme and ferrous heme reference absorption peak positions of the spectrophotometer for that buffer.

10. The spectra of the two molecules (i.e., a ferric heme molecule and a ferrous heme molecule) are different. Therefore, any spectroscopic analysis of a test stool sample which concentrates and purifies those two molecules should be very predictable regardless of the wavelength at which the spectrophotometer operates and the resulting observable differences in the absorption spectra of the two molecules should, in principle, allow the identification of the molecule present independently of the wavelength at which the spectrophotometer operates.

11. The variation with pH of the wavelengths of the absorption peaks of stool samples is negligible over a reasonably large useful pH range. To demonstrate this, I prepared reference samples of ferrous hemoglobin and ferric hemoglobin having pH values of 4.5, 6.8, 7.4 and 8.0 in essentially the same manner described on page 15, lines 9-18 of the specification, and used a

spectrophotometer to determine the locations of the main Soret absorption peaks. The results, listed in Table 1 below, show that there is no appreciable variation in the location of the Soret absorption peak of ferrous hemoglobin in the useful pH range from 6.8 to 8.0. Specifically, Table 1 shows measured wavelengths of the Soret peak locations at different pH values for ferrous heme and ferric heme. In each case there is very little change in the locations of the absorption peaks from a pH of about 6.8 to about 8.0. (Also, in each case, the peaks become difficult to differentiate at the lower pH of 4.5 .)

Table 1

Peak Wavelength of Soret Peak (nm)

Sample pH	Ferrous Hemoglobin	Ferric Hemoglobin
4.5	408.5	407.0
6.8	414.6	406.5
7.4	414.6	406.3
8.0	414.6	406.3

12. I also observed that the locations of the secondary "540 nm" and "576 nm" absorption peaks were not significantly changed for pH values of 6.8, 7.4 and 8.0.

13. The results in Table 1 show that the variation in the location of the Soret absorption peak of ferric hemoglobin in the useful pH range from 6.8 to 8.0 is only 0.2 nanometers. This variation is not appreciable with respect to the present invention, because 0.2

nanometers is negligible compared to the much larger difference of approximately 8 nanometers in the location of the Soret absorption peaks of ferrous hemoglobin and the location of the Soret absorption peaks of ferric hemoglobin in the pH range from 6.8 to 8.0. The locations of the Soret absorption peaks of 408 nanometers for ferrous hemoglobin and 415 nanometers for ferric hemoglobin set forth on page 14 of the specification for a pH of 7.4 are equally applicable for samples having a pH anywhere in the range from 6.8 to 8.0. Performing the invention as claimed provides the same results regardless of whether absorption spectra of the test stool sample are compared with the disclosed reference values corresponding to a pH of 7.4 or with absorption spectra of ferric heme and ferrous heme reference samples prepared in essentially the same manner as the test stool sample.

14. The nitrocellulose sample filter described in the above application selectively binds and concentrates the heme material of the test sample so that it can be measured with any spectrometer, irrespective of the wavelength range of the spectrophotometer. The wavelength range of the spectrometer would not make a significant difference, and there is no practical reason why the spectrophotometer cannot be an IR spectrophotometer. The identification of ferrous heme and ferric heme based on differences in observed spectra in principle does not depend on the particular wavelength at which the difference in spectra is found.

15. Anyone skilled in this art who had read the above application could easily have prepared reference samples including ferric heme and ferrous heme in essentially the same manner as the test stool sample, determined the corresponding reference absorption peaks for the ferric heme and ferrous heme reference samples using an IR spectrophotometer, obtained the

spectra of the test stool sample, and thereby readily determined by comparison of the test stool spectra and the reference sample spectra whether the stool test sample contained relative amounts of ferrous heme and ferric heme that were indicative of whether the heme in the test stool sample originated in the upper or lower GI tract.

16. Some of the hemoglobin breakdown products, including porphyrin, that are inherently included in the spectroscopic measurement in accordance with the claimed invention have lost their ability to have peroxidase activity. When samples of such breakdown products are acidified, they convert all of the ferrous heme to ferric heme.

17. The specification of the above application describes using conventional spectroscopy to evaluate the chemical condition of iron protoporphyrin rings of molecules that can be in a range of compounds ranging from native hemoglobin to heme which has been highly degraded by protease activity. The hemoglobin molecule has a substantial amount of protein folded around it which can be degraded a great deal without significantly affecting absorption spectra of the iron protoporphyrin ring. The iron protoporphyrin ring is the part of hemoglobin module that holds an iron molecule. The main absorption peaks are caused by the iron protoporphyrin ring part of the hemoglobin molecule and the degraded derivatives of hemoglobin molecules. Consequently, as long as the porphyrin ring is still present, the absorption spectra of hemoglobin degradation products are essentially unchanged from those of the native hemoglobin molecule.

18. The patent application describes determining the spectra of the iron

protoporphyrin ring to measure whether the heme in the test stool sample is ferrous heme or ferric heme. The spectra indicate relative amounts of ferric vs. ferrous porphyrin rings which are part of the hemoglobin molecule or of a number of hemoglobin degradation products. The spectra is a summation of the spectra for ferric heme and ferrous heme, and the relative amounts can be determined by mathematical methods as set forth on pages 20 and 21 of the specification to determine the fraction of the individual spectra needed to cause the observed spectrum, wherein in the equation shown on page 20, x_1 indicates the amount of ferrous heme and x_2 indicates the amount of ferric heme. Those quantities can be obtained by solving the system of equations shown on page 21, and the relative amounts of ferrous and ferric heme then can be obtained as a ratio between x_1 and x_2. Spectra are typically plotted as absorption versus wavelength, as in Fig. 2 of the specification, and those skilled in the art typically are able to visually determine the presence of ferric or ferrous forms by pattern recognition. For example the presence of the peaks at 540 nm and 576 nm are easily recognized features of ferrous forms of the molecule. The presence of a broad Soret band or a Soret band with a dip in the middle is recognized as a mixture of ferrous and ferric forms. The heights of the 540 nm peak or the 576 nm peak relative to the height of the Soret band may also serve as a visual cue for a mixture of ferrous and ferric forms. The position of the Soret band can also be visually determined to indicate a predominance of ferric or ferrous forms.

19. Hematin is included in <u>but is not specific to</u> the ferric heme detected by the present invention. In contrast to the subsequently described Fielder reference, the present invention does not use detection of hematin as the differentiating factor between upper and lower GI bleeds. The presence of such additional hemoglobin breakdown products cannot be determined by measuring

hemoglobin using the peroxidase activity test disclosed in the subsequently described Fielder reference.

20. In the spectroscopic analysis described in the above application, the amount of hematin alone is not measured, and instead the presence of hematin and a number of hemoglobin breakdown products in addition to hematin are simultaneously measured. The spectroscopic analysis of the present invention looks at the iron protoporphyrin rings of hemoglobin molecules and additional hemoglobin breakdown products which may include hematin.

21. The method of the present invention detects the presence and the relative amounts of all ferric hemes, including ferric hemes other than hematin. The measured degradation of hemoglobin is dependent primarily upon acidic exposure of the hemoglobin in the stomach and is not significantly affected by bacterial action in the lower GI tract. The spectroscopic measurement in the present invention is dependent primarily upon presence or absence of acidic exposure of hemoglobin in the stomach and is not significantly affected by bacterial action in the lower GI tract. Consequently, the method of the present invention is believed not to exhibit an erroneous indication of an upper GI bleed due to hemoglobin products formed by bacterial action that could give an erroneous indication of an upper GI bleed in the method of Fielder, and therefore is more reliable than the method described in the Fielder reference.

22. The Fielder reference discloses a method for indicating the presence of blood originating in the lower GI tract. The pH of a liquid stool sample is lowered to a value of 3.5 to 5.0 using an acidic buffer in order to cause precipitation of hematin. The resulting solution is

filtered or centrifuged to remove any precipitated hematin. The filtrate or supernatant is tested for peroxidase activity to determine the presence or absence of hemoglobin. A positive result is interpreted to indicate bleeding in the lower GI tract. The Fielder reference does not disclose any test for providing a positive indication of blood originating in the upper GI tract.

23. The pH of the solution used to prepare the test stool sample in the Fielder reference is very low, i.e., 3.5-5.0, and this would cause conversion of most of the ferrous heme to ferric heme and therefore would tend to artifactually reduce or eliminate the presence of ferrous heme in the sample. The acid environment to which the Fielder stool sample is subjected during preparation greatly compromises the ability of the Fielder method to provide a reliable conclusion that the subject stool sample has not been exposed to the acid environment of the stomach.

24. A problem with the Fielder approach is that not all hemoglobin is converted by stomach acid to hematin in the upper GI tract. It is well known that the degradation of hemoglobin from an upper GI bleed is highly variable. (Goldschmidt MD, Ahlquist DA, Wieand HS, McGill DB, et. al. Measurement of degraded fecal hemoglobin-heme to estimate gastrointestinal site of occult bleeding: appraisal of its clinical utility. Digestive Diseases and Sciences. 1988. 33:605-608.) As indicated on page 13, lines 5 and 6 of the specification of the above identified patent application, some hemoglobin caused by bleeding in the upper GI tract passes unchanged into the lower GI tract, and could be detected by the peroxidase test of the Fielder reference and could cause a false positive identification of a lower GI bleed.

25. The Fielder technique of measuring the peroxidase activity in the supernatant is capable of only inaccurately indicating whether bleeding originated in the lower GI tract, but is not capable of determining whether bleeding occurred in the upper GI tract. In Fielder, only the presence of a significant amount of precipitated hematin would indicate the presence of an upper GI bleed, but there is no indication in Fielder that constitutes a sufficient amount of precipitated hematin to indicate an upper GI bleed.

26. A lower GI tract bleed may result in the production of hematin through the action of bacteria and the in vitro exposure to acid employed by the Fielder method. The formation of hematin from hemoglobin requires acid exposure and bacterial action. Fielder depends upon the conversion of hemoglobin which may occur at highly variable rates that depend upon the amount of time the hemoglobin is present in the patient's gut and in the amount of bacteria present. In the present invention, only the result of the acid exposure of hemoglobin in the stomach is measured, and that result is independent of the highly variable amount of conversion of hemoglobin to hematin. Consequently, when a large amount of bacterial degradation of hemoglobin occurs, the method disclosed in the Fielder reference could give a false positive indication of upper GI tract bleeding and fail to indicate the lower GI tract bleeding, which is the most serious type of error because it could result in failure to diagnose cancer causing the bleeding in the colon.

27. In the described invention, the solid stool sample is put into buffer solution to achieve a very low salt level and to eliminate fecal particulate matter and bacteria from the sample. Since the resulting ionic strength is low, the blood cells in the test sample burst and release many of the hemoglobin breakdown products into the solution so that they can effectively

bind to the nitrocellulose filter.

28. Although hemoglobin is one of a number of substances that have peroxidase activity, peroxidase activity is not specific to hemoglobin. The Fielder reference depends upon the peroxidase activity of hemoglobin. However, the presence of various hemoglobin degradation products in a stool test sample cannot be determined by using the peroxidase activity test disclosed in the Fielder reference.

29. A significant problem with the Fielder method is that various other substances have peroxidase activity, including dietary animal myoglobin or vegetable peroxidases (e.g., horseradish peroxidase). Therefore, testing for peroxidase activity can result in false positives which erroneously indicate a lower GI bleed and therefore lead to costly and unnecessary follow-up evaluations.

30. The step of "determining a sample absorption spectra of the stool sample" in the present invention results in determining the absorption spectra of hemoglobin and also of a number of hemoglobin breakdown products because the spectroscopic analysis in effect measures the oxidation state of the iron protoporphyrin rings regardless of the amount of breakdown of the original hemoglobin molecule and therefore measures the heme iron protoporphyrin rings not only of the hemoglobin detected by the peroxidase activity test of Fielder, but also of the hematin that ordinarily would be discarded by the Fielder method and also measures various other hemoglobin degradation products that would not be detected by the Fielder method.

31. Measurement of the spectra as described in the present application to indicate the presence of both/either ferrous heme or ferric heme could not be accomplished with Fielder's stool sample because it has been acidified to a pH of 3.5 to 5.0 during preparation of the stool sample. The acidification converts most of the ferrous heme to ferric heme and therefore precludes an accurate characterization of the sample by spectroscopy.

32. People skilled in this art ordinarily would not have referred to a published document to obtain reference values for absorption peaks of ferric heme and ferrous heme with which to compare the spectra of a test stool sample. This is because people skilled in the art wanting to use spectroscopy to measure the presence of ferric heme and/or ferrous heme in a stool sample would have prepared reference samples of ferric heme and/or ferrous heme at the same pH as the test stool sample and measured the ferric heme and/or ferrous heme reference samples to obtain their reference absorption peaks.

33. The Machida reference discloses using the nitrocellulose filter to bind antibody molecules. The solution then is allowed to move across the nitrocellulose filter by capillary action or absorption, and if there is hemoglobin in the solution, it selectively binds to the antibody that is already bound to the nitrocellulose.

34. The method disclosed in the Machida reference would not work in the present invention because the antibody disclosed therein is specific for hemoglobin and would not bind the ferric heme breakdown products. The antibody referred to in Machida would bind too little of the hemoglobin to be easily detected by spectrophotometry.

35. The Schmitz reference does not refer to using TE buffer in preparing a stool sample and it does not refer to any advantage of using TE buffer in preparing a stool sample. The Schmitz reference does not disclose any advantage to mixing the stool sample with TE buffer to provide a lower ionic strength than typical "biological conditions" and thereby cause red blood cells to swell and burst and thereby release the hemoglobin and its degradation products into solution.

36. The Hydrofluor-Combo reference discloses a dehydration treatment involving exposure of cellulose acetate filters to increasing concentration of alcohol in 5% glycerol, followed by using 2% 1,4 diazabicyclo [2.2.2] octane mixed in greater than 95% glycerol combined with a 20 minute heating treatment to clarify the filter. However, the Hydrofluor-Combo reference does not disclose simply using glycerol in a buffer to clarify a filter, and only discloses a more involved procedure also including the octane and heating. I discovered that highly diluted glycerol was not effective. The procedure disclosed in the Hydrofluor-Combo reference would not be suitable for the present invention due to the need for a heat treatment, the high viscosity of the final reagent, and the amount of time required. Only after trial and error, including experiments with other solvents and experiments in which nitrocellulose filters were wetted with a range of glycerol concentrations and the filter translucency measured did I find a suitable concentration of glycerol that was effective for making the sample filter more transparent in accordance with the present invention.

37. Ferric hemes other than hematin which are not precipitated also are formed in the upper GI tract. Consequently, there can be an upper GI tract bleed which results in ferric hemes

in the stool that are not precipitated and therefore are capable of being detected by the Fielder method and erroneously indicating a lower GI bleed.

38. Brady discloses spectral peak information for the enzyme L-tryptophan-2,3-dioxygenase, but it is not evident that absorption peaks found by Brady for the enzyme L-tryptophan-2,3-dioxygenase would be the same as those for hemoglobin or hemoglobin derived products because they are completely different molecules.

39. An extraction procedure which includes exposure to acid as taught by Hacker would destroy the ferrous forms of heme (through exposure to glacial acetic acid) and would not allow for their measurement which is a key component of the described method of the present invention.

40. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that all my statements are made with the knowledge that false statements are punishable by fine or imprisonment under Section 1001 of Title 18 of the United States Code.

4-1-04 Brian L. Craine

Date Brian L . Craine